The Destabilization of IL-2 mRNA by a Premature Stop Codon and Its Differential Stabilization by Trans-Acting Inhibitors of Protein Synthesis Do Not Support a Role for Active Translation in mRNA Stability

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To investigate the role that translation plays in the stabilization of the IL-2 mRNA, we inhibited protein synthesis in both cis and trans. To block translation in trans, we utilized the inhibitors puromycin (PUR) and cycloheximide (CHX), which differentially affect polysome structure. We found that CHX enhances the stability of IL-2 mRNA in cells stimulated with anti-TCR Ab alone, but it inhibits CD28-induced message stabilization in costimulated cells. In contrast, PUR had a minimal effect on IL-2 mRNA stability in either the presence or absence of costimulation. The differential effects of these two inhibitors suggest that: 1) CHX is unlikely to stabilize the IL-2 mRNA by inhibiting the expression of a labile RNase; 2) CD28-mediated IL-2 mRNA stabilization does not require translation; and 3) IL-2 mRNA decay is not coupled to translation. To block translation in cis, we generated sequence-tagged IL-2 genomic reporters that contain a premature termination codon (PTC). In both the presence and absence of costimulation, these PTC-containing mRNAs exhibit drastically diminished stability. Interestingly, the addition of CHX but not PUR completely restored CD28-mediated stabilization, suggesting that CHX can block the enhanced decay induced by a PTC. Finally, CHX was able to superinduce IL-2 mRNA levels in anti-TCR Ab-stimulated cells but not in CD28-costimulated cells, suggesting that CHX may also act by other mechanisms. The Journal of Immunology, 1999, 163: 3231–3330.

The IL-2 mRNA, like many cytokine and proto-oncogene mRNAs, contains several copies of an AU-rich sequence element (ARE) within its 3’ untranslated region (UTR). In heterologous systems, the ARE has been shown to shorten mRNA half-life in a cis-dominant fashion (1). The message lability conferred by the ARE is likely to be an important regulatory component governing the expression of these genes as it is highly conserved throughout evolution (2). Recent findings in a knockout mouse strain, lacking a protein that binds the ARE of the TNF-α mRNA, provide in vivo evidence for this concept. These mice spontaneously develop autoimmune disease that is associated with a prolonged TNF-α mRNA half-life and elevated secretion of TNF-α (3). Interestingly, a number of tumor cell lines have also been identified in which elevated expression of a cytokine or proto-oncogene mRNA is a consequence of a deletion of their 3’ UTR instability element (4–6).

Early studies on the regulation of cytokine and proto-oncogene expression first raised the possibility that ARE-mediated mRNA decay is coupled to translation (7–11). Studies on cytokine gene expression found that drugs that inhibit protein synthesis superinduce mRNA levels without increasing transcription (12–14). This increase in mRNA was partially attributable to an increase in the half-lives of these mRNAs (12, 14). For a subset of ARE-containing mRNAs (e.g., IL-2), physiologic stimuli, such as costimulation through the CD28 receptor on T cells, also prolong mRNA half-life (14, 15). However, earlier studies with protein synthesis inhibitors did not examine their effects on CD28-mediated mRNA stabilization.

Various hypotheses have been proposed to explain how inhibition of translation may stabilize some mRNAs (16, 17). One is that mRNA degradation is coupled to translation; thus, blocking translation prolongs mRNA half-life. Another hypothesis is that degradation is dependent on a labile protein whose synthesis is blocked by the translational inhibitor. In addition, some translational inhibitors, such as cycloheximide (CHX), cause ribosomes to “freeze” on the mRNA, potentially shielding it from degradation by cytoplasmic RNases (10, 18, 19). A number of studies have attempted to distinguish between these possibilities, with contradictory results (20–24). For example, Koeller et al. used the 5’ UTR of the ferritin mRNA to control, in an iron-dependent fashion, the translation of a chimeric mRNA containing the ARE from the c-fos mRNA (21). They found that mRNA stability is not related to translation of the message. In contrast, Winstall et al., using the same ferritin mRNA 5’ UTR and the ARE from the c-fos mRNA, reached the opposite conclusion (24). While the role of translation in mRNA stability remains unresolved, there has been little dispute that certain protein synthesis inhibitors stabilize these short-lived mRNAs.

Recently, it has been reported that in the absence of costimulation IL-2 mRNA is found predominantly in the soluble fraction of the cytoplasmic mRNA pool rather than associated with polysomes (25). These authors suggested that costimulation may control IL-2 expression at the translational level. Their observations prompted us to examine what role translation may have if, any, in the CD28-mediated stabilization of the IL-2 mRNA. To do so, we

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2 Abbreviations used in this paper: ARE, AU rich sequence element; CHX, cycloheximide; CSA, cyclosporin A; PTC, premature termination codon; PUR, puromycin; UTR, untranslated region; JNK, c-Jun NH2-terminal kinase.
blocked translation in trans using CHX or puromycin (PUR), two inhibitors of protein synthesis that differ mechanistically (18). The use of CHX causes stabilization of polyosomes, in contrast to PUR, which disrupts polyosomes. While CHX has previously been shown to stabilize and superinduce IL-2 mRNA, the effects of PUR on IL-2 mRNA have not been examined (9, 12, 14, 18). We reasoned that if these two translational inhibitors had differential effects on IL-2 mRNA, it would indicate that inhibition of translation, in and of itself, was not the mechanism by which the message was stabilized.

To further distinguish between the global effects that these inhibitors have on protein synthesis and disruption of IL-2 mRNA translation per se, we sought to selectively block translation of the IL-2 mRNA by introducing a premature termination codon (PTC) into exon 1. This in turn required the development of a bona fide reporter system. We have recently described such a system, in which the reporter, under the control of the IL-2 promoter, has been stably introduced into a normal mouse CD4+ T cell clone (26). This allows transcription to be induced by TCR stimulation and to be selectively inhibited with cyclosporin A (CSA) when and to be selectively inhibited with cyclosporin A (CSA) when CD28-mediated mRNA stabilization. Interestingly, CHX stabilized the PTC-containing IL-2 reporter mRNA in both the presence and absence of CD28 costimulation. These results suggest that translation does not play a critical role in determining IL-2 mRNA stability.

Materials and Methods

Reporter constructs

Construction of the IL2X reporter is described elsewhere (26). The sequence-tagged IL-2 genomic construct containing a PTC in exon 1 (IL2Nt) was generated in the same fashion as IL2X except for the XhoI linker (no. 1128, New England Biolabs, Beverly, MA) was used instead of a XhoI linker. This results in a frameshift producing multiple PTC, the first of which occurs at codon 62. The IL2NtpA reporter was constructed by substituting the 3' portion of the IL2XpA construct (26) for the corresponding restriction fragment in the IL2Nt reporter.

Cell culture, transfection, and stimulations

Establishment of the stable transfectants, maintenance of the A.E7 cultures, and T cell activation were as described previously (26). In brief, 2 × 10^6 cells were stimulated with plate-bound anti-TCR-β Ab H57-597 (29) in the presence or absence of the anti-CD28 mAb 37.51 (a gift from Dr. J. Allison, University of California, Berkely, CA). Both Abs were used at a concentration that elicited maximal IL-2 secretion from the A.E7 cells. Cells were stimulated for the indicated length of time, after which both the cells and supernatants were harvested. To block IL-2 transcription, CSA (Calbiochem, La Jolla, CA) was added to a final concentration of 0.5 μg/ml after 3 h of stimulation. To block translation, CHX (Sigma, St. Louis, MO) was added to a final concentration of 25 μg/ml or PUR (Sigma) was added to a final concentration of 10 μg/ml after 3 h of stimulation. The concentrations at which all three drugs were used completely blocked IL-2 expression in CD28-costimulated cells when added individually at the zero time point. Under the same conditions, CHX and PUR inhibited incorporation of L-[35S]methionine into total protein by 95% and 75%, respectively (data not shown). PUR concentrations (>40 μg/ml) that inhibited total protein synthesis by 95% gave the same results as those obtained at 10 μg/ml (Fig. 1C and data not shown). Inhibition of protein synthesis had no effect on H-2K mRNA levels during the time span of these experiments (data not shown).

RNA isolation and quantitation

Cytoplasmic RNA was prepared by lysing cells on ice in 50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl2, and 0.5% NP40 with 1000 U/ml of RNase inhibitor (5′→3′, Boulder, CO). Nuclei were pelleted at 300 × g for 5 min at 4°C. The supernatant was then denatured in a guanidinium isothiocyanate buffer and RNA was purified by binding to a silica-based matrix in a 96-well format according to the manufacturer's instructions (Qiagen, Santa Clarita, CA). RT-PCR reactions were performed in the same tube using 10–100 ng of total RNA in a× TaqMan EZ buffer, 2.5 mM manganese acetate, 300 μM of each dNTP, and 100 U/ml of rTth DNA polymerase in a total volume of 25 μl. Alternatively, coupled RT-PCR reactions were performed in a single tube using Moloney murine leukemia virus reverse transcriptase (0.25 U/ml) and AmpliTaq Gold DNA polymerase (0.025 U/ml) in a× TaqMan buffer A and 5.5 mM magnesium chloride (Applied Biosystems, Foster City, CA). Detection of the amplicon was achieved by dequenching of a 6-FAM-labeled IL-2-specific probe during amplification and measurement of the emitted fluorescence with an ABI7700 sequence detection system (Applied Biosystems, Foster City, CA). Primers, probe, and reaction conditions for detection of the IL-2, IL2X, and H-2K mRNAs were as specified earlier (26). These primers will only amplify fully spliced mRNA. In pilot experiments using primers that detect unspliced mRNA (and DNA), we found that nuclear contamination of the cytoplasmic fraction was minimal or nonexistent (results not shown). The IL2Nt and IL2NtpA mRNAs were detected by RT-PCR as described for the IL2X mRNA except for the use of a forward primer positioned in exon 1 that is specific for these constructs (5′-TGGACCTACAGGTTGCGG-3′) and a different IL-2-specific back primer (5′-TGGCCTGCTTGGGC-3′) that is positioned at the exon 2/3 junction. This back primer was also used to prime the reverse transcription step. These primers will only amplify fully spliced mRNA. The probe was the same as that used for the IL-2 and IL2X mRNAs (26). The RT-PCR reaction mixture and conditions were the same except that the PCR extension step was conducted at 58°C. For IL2X and H-2K sequence-tagged IL-2 mRNAs with total RNA from the relevant stable transfectant or parental A.E7 cells costimulated with anti-CD28 mAb for 4 h. The log of the total RNA (ng) plotted vs the threshold cycle number is a linear function. The threshold cycle number is defined as the amplification cycle number at which the fluorescence emitted is >10 SD above the average baseline fluorescence (usually the amount of fluorescence measured between cycles 3 and 15). In general, the standard curves were linear over the range of 5 pg to 100 ng of total RNA. The relative amount of IL-2 or IL2X reporter mRNA in the unknown samples was determined from the standard curves, corrected for the amount of H-2K mRNA present, and the corrected values were normalized to the value of the 3-h sample stimulated with anti-TCR alone. All samples were assayed in triplicate; the arithmetic means and SEM are shown in the figures and in Table I.

Results

Differential stabilization and superinduction of IL-2 mRNA by CHX and PUR

To examine the role of translation in CD28-mediated IL-2 mRNA stabilization, we first performed a comparative analysis of the effect of CHX and PUR on IL-2 mRNA levels in T cells that were stimulated through their TCR alone. To measure IL-2 mRNA half-life, transcription was blocked by adding CSA. In Fig. 1A, T cells were stimulated with anti-TCR Ab for 3 h before the addition of PUR, CSA, or CSA plus PUR. The addition of PUR alone had little or no superinductive effect on IL-2 mRNA levels (Δ). In CSA-treated cells (□), the addition of PUR (▲) did not appreciably alter the decay of the IL-2 mRNA (mean t1/2 = 30 vs 29 min;
Table I. Effect of translational inhibition on mRNA stability

<table>
<thead>
<tr>
<th>Translational Inhibitor</th>
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<tr>
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<td>TCR</td>
<td>TCR + CD28</td>
<td>TCR</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>(±5, n = 3)</td>
<td>26</td>
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<tr>
<td></td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(±3, n = 4)</td>
</tr>
<tr>
<td>CHX</td>
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<td>(±6, n = 2)</td>
<td>52</td>
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<tr>
<td></td>
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<td>(n = 3)</td>
<td>(±7, n = 3)</td>
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<tr>
<td>PUR</td>
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a Mean mRNA ± in min ± SEM.
b No decay of mRNA between 3 and 5 h.
c See text for explanation of longer NtpA ±.

As observed with anti-TCR Ab alone, the addition of PUR to cells that had been costimulated for 3 h with anti-CD28 Ab had little or no effect on steady-state IL-2 mRNA levels (Δ, Fig. 2A). As reported elsewhere (15, 26) and as shown in independent experiments in Fig. 2, IL-2 mRNA levels are transiently stabilized in CD28-costimulated cells (●) between 3 and 5 h, relative to cells stimulated with anti-TCR Ab alone (■, Fig. 1). The addition of PUR did not affect CD28-mediated IL-2 mRNA stabilization (▲, Fig. 2A), demonstrating that translation is not necessary for such stabilization.

Quite different results were obtained when CHX was added to cells that had been costimulated with anti-CD28. In contrast to T cells stimulated with anti-TCR Ab alone, the addition of CHX did not superinduce IL-2 mRNA above the level found in CD28-costimulated cells (Δ, Fig. 2B). Furthermore, the addition of CHX to CSA-treated cells (▲, Fig. 2B) partially inhibited CD28-mediated IL-2 mRNA stabilization (mean t½ = 107 min, Table I). In CD28-costimulated cells treated with CSA alone, IL-2 mRNA did not decay during the same interval (●, Fig. 2B).

### FIGURE 1. Effects of translational inhibitors on IL-2 mRNA stability in TCR-stimulated cells. IL-2 mRNA quantitation and normalization are as described in Materials and Methods. Values are the arithmetic mean of triplicate samples, and SEs of the mean are shown. Where error bars are not visible, the size of the bar was smaller than the figure symbol.

A. Relative level of the IL-2 mRNA in A.E7 cells stimulated with anti-TCR Ab alone (■) for 3 h before the addition of CSA (■), 10 μg/ml PUR (▲), or CSA plus PUR (▲). Subsequently, RNA was harvested at the indicated times from CSA-treated (▲, ▲) and untreated (Δ, Δ) cells. Results from a single experiment are shown. B. Relative level of the IL-2 mRNA in A.E7 cells stimulated with anti-TCR Ab alone (■) for 3 h before the addition of CSA (■, ▲), CHX (Δ), or CSA plus CHX (▲). Subsequently, RNA was harvested at the indicated times from CSA-treated (▲, ▲) and untreated (Δ, Δ) cells. Results shown are from the same experiment as in A. C. Relative level of the IL-2 mRNA in A.E7 cells stimulated with anti-TCR Ab alone for 3 h before the addition of CSA (■, ▲), CSA plus 100 μg/ml of PUR (▲), or CSA plus CHX (●). Results from a single experiment are shown.

Table I. Effect of translational inhibition on mRNA stability

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<tr>
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<td>(±6, n = 2)</td>
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a Mean mRNA ± in min ± SEM.
b No decay of mRNA between 3 and 5 h.
c See text for explanation of longer NtpA ±.
A sequence-tagged reporter mimics the endogenous IL-2 mRNA

Before testing the effects of introducing a PTC into our IL-2 reporter mRNA, we first examined the decay of the wild-type sequence-tagged IL-2 reporter (IL2X) mRNA. As we reported elsewhere (26), and as shown in an independent experiment in Fig. 3A, steady-state IL2X mRNA levels in CD28-costimulated cells (●) are greater than in cells that have been stimulated with anti-TCR Ab alone (□). In part, this is due to the enhanced stability of the mRNA in CD28-costimulated cells (●) between 3 and 5 h, relative to cells stimulated with anti-TCR Ab alone (□, t1/2 = 20 min). In our earlier report, we demonstrated that IL2X reporter mRNA levels parallel that of the endogenous IL-2 mRNA (26). The effects of CHX and PUR on IL2X mRNA stability are shown in Fig. 3B. As was observed for the endogenous IL-2 mRNA in cells stimulated with anti-TCR Ab alone and treated with CSA (Fig. 1), the addition of CHX (●), but not PUR (▲), stabilizes the IL2X mRNA (t1/2 = 73 vs 24 min). The difference in IL2X mRNA levels between CSA-treated and CSA plus PUR-treated cells in this experiment at 5 h was not observed in several other experiments. Thus, the effects of CHX and PUR on decay of the wild-type sequence-tagged IL-2 reporter mRNA mimic those observed with the native IL-2 mRNA.

Premature translational termination abolishes CD28-induced mRNA stabilization

To directly test the effect of translation on IL-2 message stability, we sought to selectively block translation of the mRNA by generating a sequence-tagged IL-2 reporter construct with a PTC in the first exon (IL2Nt). The consequence of introducing a PTC on CD28-mediated mRNA stabilization is shown in Fig. 4A. This stabilization, which is ordinarily observed for the IL-2 and IL2X mRNAs in the presence of CSA (Figs. 2 and 3A), is no longer seen with the IL2Nt mRNA, which has a mean half-life of 24 min in CD28-costimulated cells (●, Fig. 4A; Table I). In contrast, decay of IL2Nt mRNA in cells stimulated through the TCR alone (■, Fig. 4A) appears similar (mean t1/2 = 26 min) to that of the IL-2 and IL2X mRNAs under the same conditions (■, Figs. 1 and 3B; Table I). This last observation suggests that the PTC present in the IL2Nt mRNA did not result in enhanced degradation of the mRNA. However, these results could be explained by three alternative mechanisms that are not mutually exclusive. One is that premature translational termination prevents CD28-mediated stabilization of IL-2 mRNA. The second is that CD28 signals increase the decay of mRNA containing nonsense codons. Third, premature translational termination could decrease the cytoplasmic stability of the IL-2 mRNA, but it is not apparent in these experiments because of the rapid rate of decay already superimposed by the 3′ ARE.

To determine whether mRNA decay induced by premature translational termination was being masked in the IL2Nt mRNA by ARE-mediated decay, we substituted the 3′ UTR and poly(A) signal from the late region of SV40 for the 3′ UTR and poly(A) signal of IL-2 present in IL2Nt. This construct was designated IL2NtpA. Such a substitution in the wild-type IL2X construct increases the mRNA half-life in anti-TCR-stimulated cells treated with CSA from 23 min to >300 min (26). In contrast, the mean half-life (47 min) of the IL2NtpA mRNA (■, Fig. 4B) increased <2-fold relative to that of the IL2Nt mRNA. The IL2NtpA mRNA was also not stabilized (t1/2 = 27 min.) in CD28-costimulated cells (●, Fig. 4B). The shorter half-life of the IL2NtpA mRNA in CD28-costimulated cells relative to cells stimulated with anti-TCR Ab alone was also observed with the IL2XpA mRNA (26). In that case, the diminished stability was found to be the result of a second instability element within the mRNA coding region that is active

FIGURE 2. Effects of translational inhibitors on IL-2 mRNA stability in CD28 costimulated cells. The relative level of the endogenous IL-2 mRNA in Nt/A.E7 stable transfectants stimulated with anti-TCR Ab plus anti-CD28 Ab is shown in both panels. Values are the arithmetic mean of triplicate samples, and SEs of the mean are shown. Where error bars are not visible, the size of the bar was smaller than the figure symbol. A, Cells were stimulated with anti-TCR Ab plus anti-CD28 Ab (●) for 3 h before the addition of CSA (▲), PUR (△), or CSA plus PUR (▲). Subsequently, RNA was harvested at the indicated times from CSA-treated (▲, ●) and untreated (△, ○) cells. Results from a single experiment are shown. B, Cells were stimulated with anti-TCR Ab plus anti-CD28 Ab (●) for 3 h before the addition of CSA (▲), CHX (△), or CSA plus CHX (▲). Subsequently, RNA was harvested at the indicated times from CSA-treated (▲, ●) and untreated (△, ○) cells. Results from a single experiment are shown.
only in CD28-costimulated cells. These results, summarized in Table I, demonstrate that cytoplasmic IL-2 mRNA stability is severely reduced by premature termination of IL-2 mRNA translation and that CD28-induced stabilization of ARE-mediated mRNA decay is abolished.

Differential stabilization and superinduction of IL2Nt mRNA by CHX and PUR

We assessed the effects of CHX and PUR on the IL2Nt mRNA, whose translation is already blocked in cis, to ascertain whether there might be additive effects on mRNA stability in our system. PUR had little effect on IL2Nt mRNA levels (Δ, Fig. 5A) or stability (mean t1/2 = 34 min) in cells stimulated with anti-TCR Ab alone (▲, Fig. 5B; Table I). Interestingly, the addition of CHX to cells stimulated with anti-TCR Ab alone superinduces IL2Nt mRNA levels (○, Fig. 5A) by nearly the same relative extent as the IL-2 mRNA (Δ, Fig. 1B). Superinduction of the IL2Nt mRNA, as for IL-2 mRNA, is at least partially attributable to stabilization of the mRNA (mean t1/2 = 52 min) in cells stimulated with anti-TCR Ab alone (●, Fig. 5B; Table I).

To help distinguish whether CHX is acting to stabilize the IL2Nt mRNA primarily by blocking ARE-mediated or PTC-mediated decay, we tested the effect of CHX on the decay of IL2NtpA mRNA. As described above, this mRNA is degraded fairly rapidly, even though it contains a stable SV40 3′ UTR. The addition of CHX stabilized (t1/2 = 186 min) the IL2NtpA mRNA in CSA-treated cells (●, Fig. 5C). This observation supports the hypothesis that CHX acts to stabilize the IL2Nt mRNA largely by blocking PTC-mediated decay. These results are summarized in Table I. Thus, it appears that stabilization of mRNA on polysomes by CHX is sufficient to retard mRNA decay, even if the ribosomes can only bind to the 5′ end of the message as is the case for the IL2Nt mRNA.

Finally, we examined what effect protein synthesis inhibitors might have on the IL2Nt mRNA in CD28-costimulated cells. Anti-CD28 (●, Fig. 6; Table I) had little or no effect on IL2Nt mRNA stability (mean t1/2 = 24 min) as seen here and in Fig. 4A. The addition of PUR did not superinduce IL2Nt mRNA levels (△, Fig. 6A) nor did it increase the stability (▲) of the message (mean t1/2 = 25 min). In contrast, CHX almost completely restored the ability of CD28 costimulation to stabilize (mean t1/2 = 150 min) the IL2Nt mRNA (▲, Fig. 6B; Table I). The small superinductive effect of CHX on IL2Nt mRNA (△, Fig. 6B), which was not observed with the IL-2 mRNA in CD28-costimulated cells (△, Fig. 2B), is probably due to blocking degradation induced by the PTC. These results, summarized in Table I, indicate that in the presence of CHX, CD28 costimulation can function to stabilize an IL-2 mRNA whose translation is blocked in cis.
Discussion

In our studies, we found that two translational inhibitors that differ mechanistically have distinct effects on IL-2 mRNA levels (Table I and II). While CHX stabilizes and superinduces IL-2 mRNA in anti-TCR-stimulated cells, it has a small inhibitory effect on message stability and does not superinduce message levels in CD28-costimulated cells. In contrast, PUR has little effect on IL-2 mRNA levels in the presence or absence of CD28 costimulation. The differential effects of these translational inhibitors allow some inferences to be made about the stabilizing effect of CHX on the IL-2 mRNA. It seems unlikely that CHX acts by blocking the expression of a labile RNase, because PUR should also stabilize the IL-2 mRNA if this were the mechanism. Similarly, if IL-2 mRNA decay is coupled to translation, both inhibitors should act to stabilize the mRNA. However, it should be pointed out that because we used CSA to block IL-2 transcription, our conclusions about mRNA stability may not pertain to the CSA-sensitive component of CD28-mediated IL-2 mRNA stabilization (26). Collectively, our results indicate that in cells stimulated with anti-TCR alone, IL-2 mRNA stability is unrelated to active translation. Our observation that PUR does not affect IL-2 mRNA stability in CD28-costimulated cells indicates that active translation is also not necessary for CD28-induced mRNA stabilization. This conclusion is also supported by the observation that CD28 costimulation stabilizes the IL2Nt mRNA in CHX-treated cells. However, a role for translation in these processes cannot be completely excluded, because PUR does not prevent formation of the translational initiation complex. Nonetheless, it seems most likely that the differential effects of CHX and PUR on IL-2 mRNA stability are related to the dissimilar effects these inhibitors are known to have on polysome structure. Our results with the IL2Nt mRNA further illustrate these differences.

Introduction of a PTC into the IL-2 mRNA (i.e., IL2Nt) severely diminishes its cytoplasmic stability and abolishes its CD28-induced stabilization. While such mRNA destabilization has been described previously in higher eukaryotes, it is more typical of yeast (30–35). In higher eukaryotes, PTC have also been described to affect nuclear posttranscriptional events, resulting in decreased levels of cytoplasmic mRNA without altering cytoplasmic mRNA stability (33, 36, 37). In the case of the TCR and the Ig genes, nonproductive DNA rearrangements often result in transcripts containing PTC (38). For the Ig transcripts, such codons result in an accumulation of nuclear unspliced or partially spliced mRNA and a relative depletion of fully spliced nuclear mRNA, suggesting an inhibitory effect on RNA splicing (39). The down-regulatory effect of PTC on TCR transcripts appears to be dependent on the presence of an intron within the transcript but is not associated with an alteration of the ratio of unspliced to spliced nuclear mRNA. This suggests an effect on the stability of partially spliced nuclear mRNA rather than on splicing itself (40).

Thus, the IL-2 mRNA, unlike other lymphocyte mRNAs that have been extensively studied, is a member of a small group of
transcripts in higher eukaryotes whose cytoplasmic stability is reduced by premature termination of translation (33, 41). This observation could be taken, in and of itself, to indicate that IL-2 mRNA stability is dependent on translation of the mRNA. However, in light of the differential effects of CHX and PUR on IL-2 mRNA stability, and the selective ability of CHX to stabilize the IL2Nt reporter mRNA, the reduced stability of the IL2Nt mRNA is not likely to be due to the block in translation per se. CHX also partially relieves the splicing block associated with PTC containing Ig-κ pre-mRNA, while both CHX and PUR are able to restore the stability of PTC containing partially spliced nuclear TCR-β mRNA (42–44). A number of models have been proposed in an attempt to account for the diverse consequences of harboring a PTC in the mRNA of both higher and lower eukaryotes, yet the subject remains quite controversial (33, 38, 41, 45, 46).

CD28 costimulation appears to act posttranscriptionally to counter the destabilizing effect of the IL-2 mRNA ARE. It has been demonstrated that the ARE acts to enhance shortening of the mRNA poly(A) tail (47, 48). The inability of CD28 costimulation to stabilize the IL2Nt mRNA might indicate that decay of this mRNA occurs via a pathway that is mechanistically unrelated to CD28-induced mRNA stabilization. In other systems, it has been shown that mRNAs containing PTC can decay without prior shortening of the poly(A) tail through a mechanism that involves de-capping and 5′ → 3′ exonucleolytic degradation (41, 48). Therefore, if CD28 signaling acts to inhibit ARE-mediated enhancement of poly(A) tail shortening, it may be overridden by a PTC-mediated mRNA decay process.

This may also explain how CHX can restore CD28-mediated stabilization of the IL2Nt mRNA while partially inhibiting that of the IL-2 mRNA (Fig. 7). In the presence of CHX, ribosomes would be expected to stably associate with the IL2Nt mRNA only up to the PTC in exon 1 (Fig. 7A). We speculate that this distribution of ribosomes is sufficient to protect from PTC-induced 5′ → 3′ exonucleolytic degradation but is inadequate to block degradation following ARE-mediated enhancement of poly(A) tail shortening. Under these circumstances, the latter may occur by a 3′ → 5′ exonucleolytic mechanism as this route of degradation may dominate when the 5′ end of the mRNA is blocked (22, 49, 50). Thus, CHX partially stabilizes the IL2Nt mRNA in cells stimulated with anti-TCR alone by sterically blocking 5′ → 3′ exonucleolytic degradation, and CD28 signaling can further stabilize the mRNA by inhibiting ARE-mediated enhancement of poly(A) tail shortening (Fig. 7B). In contrast, ribosomes would be expected to be stably distributed over the entire protein coding region of the wild-type IL-2 mRNA in the presence of CHX, thus shielding it from degradation following poly(A) shortening (Fig. 7C). Recent reports have demonstrated that CD28-mediated stabilization requires sequences throughout the IL-2 mRNA, including the coding region (26, 51). Those findings suggest that a higher ordered IL-2 mRNA structure, formed by direct or indirect interactions between these dispersed sequences, is required for CD28-mediated stabilization.
We speculate that for the wild-type IL-2 mRNA in the presence of CHX, the distribution of ribosomes over a more extensive region of the transcript interferes with this RNA folding and thus actually inhibits CD28-mediated stabilization (Fig. 7D). However, in the case of the IL2Nt mRNA, the topographic distribution of ribosomes would be sufficiently restricted so as not to interfere with the formation of this higher ordered RNA structure, thus allowing for CD28-mediated stabilization of this mRNA (Fig. 7B).

While our primary objective was to ascertain what role translation might play in the CD28-mediated stabilization of the IL-2 mRNA, we noted that there were also differential effects on superinduction in our system (Table II). While both PUR and CHX have been reported to superinduce proto-oncogene mRNAs, the superinductive effect of PUR on the IL-2 mRNA (Fig. 1A) is quite marginal (16). We also observed that the presence of a PTC did not interfere with CHX-induced superinduction. However, CHX could not superinduce IL-2 mRNA levels above those found in CD28-costimulated cells. Superinduction by translational inhibitors may be the consequence of several effects that these drugs have on gene expression (52). As demonstrated here and shown by earlier investigators, CHX can stabilize the IL-2 mRNA, thus contributing to superinduction (12, 14). Furthermore, some translational inhibitors have additional actions that are independent of their ability to inhibit protein synthesis (16, 52). For example, the inhibitors anisomycin, and to a lesser extent CHX, can enhance signal transduction. Both have been shown to activate the c-Jun NH2-terminal kinase (JNK) (53–55). Under normal conditions, JNK activation is synergistically enhanced by CD28 costimulation (56). JNK in turn appears to contribute to CD28-mediated stabilization of the IL-2 mRNA (51). Because JNK activation is severely inhibited by CSA, which was used in our studies to block IL-2 transcription, it is unlikely that this pathway contributes to CD28-mediated IL-2 mRNA stability in our system (26, 56). However, during superinduction (i.e., in the absence of CSA), CHX-induced JNK activation could contribute significantly to mRNA stabilization. CHX has also been reported to superinduce IL-2 mRNA levels by increasing the processing and transport of nuclear IL-2 pre-mRNA by unknown mechanisms (13). Clearly CHX has several distinct effects in our system, which could be acting alone or in concert to superinduce IL-2 mRNA.

In conclusion, our studies do not support a role for active translation in CD28-mediated stabilization of the IL-2 mRNA. The effects of blocking IL-2 mRNA translation in cis can best be explained as a consequence of 5’→3’ exonucleolytic degradation secondary to the introduction of a PTC. In our CSA-treated cells, CHX most likely increases mRNA stability by freezing ribosomes on the transcript, thus physically shielding the message from degradation. The ability of CHX to superinduce IL-2 mRNA in TCR-stimulated cells, but not in CD28-costimulated cells, suggests that

Table II. Effect of CHX on mRNA superinduction

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<th>mRNA</th>
<th>CHX Induced Superinduction</th>
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<tr>
<td></td>
<td>TCR</td>
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<tr>
<td>IL-2</td>
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<td>IL2Nt</td>
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FIGURE 6. Effects of PUR and CHX on IL2Nt mRNA in CD28-costimulated cells. The DNA structure of the construct is shown above the panels. See Fig. 4 for details. mRNA quantitation and normalization are as described in the Materials and Methods. Values are the arithmetic mean of triplicate samples, and SEs of the mean are shown. Where error bars are not visible, the size of the bar was smaller than the figure symbol. A. A.E7 cells stably transfected with the IL2Nt reporter were stimulated with anti-TCR Ab plus anti-CD28 Ab (○) for 3 h before the addition of CSA (●). PUR (▲), or CSA plus PUR (▲). Subsequently, RNA was harvested at the indicated times from CSA-treated (▲, ●) and untreated (△, ○) cells. Results from a single experiment are shown. B. Cells were stimulated with anti-TCR Ab plus anti-CD28 Ab (○) for 3 h before the addition of CSA (●), CHX (▲), or CSA plus CHX (▲). Subsequently, RNA was harvested at the indicated times from CSA-treated (▲, ●) and untreated (△, ○) cells. Results from a single experiment are shown.
under these circumstances CHX could be acting along the CD28 signaling pathway.

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References


FIGURE 7. CHX enhances IL2Nt mRNA stabilization, but inhibits IL-2 mRNA stabilization in CD28-costimulated cells. A, In the case of the IL2Nt mRNA, stable association of ribosomes with the 5' end of the mRNA in the presence of CHX is sufficient to protect it from PTC, but not ARE-induced degradation. B, In CD28-costimulated cells, the IL2Nt mRNA is able to fold into a higher ordered structure necessary for stabilization. C, In CHX-treated cells, stable association of ribosomes with the IL-2 mRNA shields it from degradation in TCR-stimulated cells. D, In CD28-costimulated cells, the IL-2 mRNA cannot assume a higher ordered structure because RNA folding is sterically hindered by the presence of ribosomes. Implicit is the postulate that CD28 signaling acts primarily to stabilize IL-2 mRNA that is not being translated.
IL-2 mRNA TRANSLATION AND MESSAGE STABILITY


